STUDY OF MICRORNA-MEDIATED REGULATION OF METASTASIS IN FIBROSARCOMA

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CERTIFICATE

This is to certify that the thesis entitled "**Study of microRNA-mediated regulation of metastasis in fibrosarcoma**" submitted by **Ms. Rojali Sethy** (Roll No. 413LS2051) in partial fulfillment of the requirements for the award of **Master of Science in Life Science** to the National Institute of Technology Rourkela is an authentic and original record of research work carried out by her under my supervision.

To the best of knowledge, the work incorporated in this thesis has not been submitted elsewhere for the award of any degree.

(Dr. Bibekanand Mallick)

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ABBREVIATIONS

ACTB	ACTB β-Actin
µ	MICRO
:	RATIO
%	PERCENTAGE
µl	Micro liter
cDNA	Complimentary DNA
PCR	Polymerase Chain Reaction
mRNA	Messenger RNA
DEPC	Diethyl Pyrocarbonate
MFS	Myxofibrosarcoma
miRNA	Micro RNA
et al.	And Other
qRT-PCR	Quantative real time PCR
DMEM	Dulbecco's Modified Eagle'sMedium

Content

Abstract

microRNAs (miRNAs) are the small non-protein-coding RNA, Regulate target gene expression via transcriptional degradation or translational repression. In this study our aim was to investigate the role of miR-429 in metastasis process in fibrosarcoma and its possible mode of regulation. Microarray data analysis for fibrosarcoma, is trying to screen out the probable miRNAs and genes responsible for metastasis. After gating a novel pair of gene and miRNA, Experimental validation of selected gene and miRNA expression was carried out through qRT-PCR in fibrosarcoma cell line HT1080, as compare to normal fibroblast cell line WI-38. The role of miR-429 in metastasis was further accessed via over-expression of the miRNA in HT1080 and carrying out during in-vitro metastasis detection assay like cell migration, cell proliferation assays, By using different assay we found that miR-429 expression is decreased in Fibrosarcoma where it might be acting as tumor suppressor in cellular model, Where it prevents tumor development by negatively inhibiting oncogene KIAA0101 that induce cell proliferation, cell migration. But Over expression of miR-429 inhibits the cell proliferation and cell migration which might be helping in metastasis and ultimately contributing to lethality of Fibrosarcoma.

Key Words: non-coding RNAs miRNA, Fibrosarcoma metastasis, microarray

Introduction

Sarcomas are a heterogeneous group of mesenchymal tumors with specific molecular characteristics, and the current classification is based on their tissue of origin and its histological appearance. They are one of the most serious cancer types in the world. They are a very rare group of the mesenchymal tumor, derived from connective tissue such as -blood, bone, cartilage, dense fibrous tissue, muscle tissue and adipose tissue [Coley WB. II *Ann Surg*, 1891]. It is also known as mesenchymal neoplasm. They differ from carcinomas because of their origin, as sarcomas origin from connective tissue, and carcinoma from epithelial tissue.

Sarcomas are involved in the abnormal growth of connective tissue that includes five major steps for sarcoma development: initiation, promotion, malignant conversion, progression, and metastasis. And Metastasis is the process in which malignant tumor cell leaves the primary site and migrates to a distance site via circulatory system and lymphatic system and establishes a secondary site. Metastatic development is a very complex process and requires multiple individual steps to establish a tumor at a secondary site.

The metastatic process involves a change at the molecular level that disrupts and modifies the tumor cell-extracellular matrix interactions and cell–cell interaction. There is a movement of malignant cell from the primary tumor to distant organs through the lymphatic or hematogenous circulatory system. It involves six major steps: local invasion, intravasation, transport through circulation, extravagation, formation of micro-metastasis colonization [Stoletov K, Kato H at al, 2001]. About 10% of deaths in patients with cancer occur due to the primary tumor, but 90% of death is due to the metastasis of cancer cells [Coley WB. II *Ann Surg* 1998].

Metastasis it is a process in which the malignant tumor cell leaves the primary site and migrate to a distance site via circulatory system and lymphatic system and establish a secondary site. Metastasis in sarcoma is the combined interaction of both tumor inducers, which promote tumor development and tumor suppressors, which inhibit tumor growth [Calin et al. 2004]. There are many of molecules which are involved in metastasis like mRNAs, non-coding RNAs like miRNAs, piRNAs,miRNA is one of them. The identification of these molecules and their mechanism of involvement are under extensive investigation. Although several of these molecules, which act as either tumor suppressors or oncogenes, have been identified in model

animal genomes, and human their precise role in metastasis is not well understood. Metastasis is well understood extensively studied in cancers of epithelial origin but is not clearly understood in cancers of mesenchymal origin (sarcoma).

There are many molecule involves in metastatic process but miRNAis one of them. These are small non-protein coding RNAs, being extensively studied and may provide new insight into cancer research. miRNA are the important regulatory molecules in plants and animals. Their function is to regulate the gene expression in a variety of manners such as-translational repression, transcriptional degradation or mRNA deadenylation. From literature study about 50% of miRNA genes are located in cancer-associated genomic regions and also know as in fragile sites [Calin et al., 2004] suggesting that miRNAs may play a more important role in metastasis.

Our aim was validation of expression of the miR-429 and its target gene KIAA0101 obtained from microarray analysis of myxoidfibrosarcoma tissues by using fibrosarcoma cell line HT1080 and establish the role of the miRNA in metastasis.

To detect the expression of selected miR -429 and KIAA0101 (qRT-PCR) was carried out in fibrosarcoma cell lines (HT-1080) as a test sample and normal fibroblast cell (WT-38). We found that miR-429 was down-regulated and KIAA0101 is over-expressed. To further establish their role in metastasis different assays were performed such as MTT to check cell viability, proliferation and migration assay.

Review of literature

Sarcomas are a rare heterogeneous family of malignant neoplasm and they share presumptive origins from the mesoderm or endoderm. However most of the origin of sarcomas remains unclear [MackalL CL, Meltzer PS, Helman LJ (2002)].

Sarcomas are divided into two types: based on the origin

1.**Soft tissue sarcoma**- These arise from connective tissues such as muscles, fat, nerves, and fibrous tissue blood vessels . About 80% of sarcomas are occur in soft tissues.[World Health Origination] on the basis of occurrence ,soft tissue sarcoma occupies a 23rd position among all cancer.Based on the origin of cancer there are many types of soft tissue sarcoma. The cells of sarcoma look different from each other under a microscope.

Table1. types of sarcoma based on their origin:

Types of sarcoma	**Cells forming tumor**
Angiosarcoma	lymphatic vessel or Blood
Chondrosarcoma	Cartilage cells
Gastrointestinal stromal tumor tract	Gastrointestinal tract
Fibrosarcoma	Fibrous tissue
Leiomyosarcoma	Gastrointestinal tract
Liposarcoma	Fat tissue
Malignant peripheral nerve sheath tumor	Nerve and Spinal Cord

Fibrosarcoma:

It is a mesenchymal tumor that origin from the fibrous tissue, Fibrosarcomas are a form of solid tumors and are different grades according to the degrees of differentiation: low grade that is differentiated, intermediate grade and high grade (anaplastic). Depending on this differentiation, the tumour cells may resemble mature spindle-shaped fibroblasts that secrete collagen. High

grade fibrosarcomas mostly shows metastasis that is they have the capacity to enter into local tissues thus spreading tumors to different body parts. Majority of deaths in fibrosarcoma is mainly due to metastasis which is poorly diagnosed or poorly understood.

2.**Osteosarcoma:** It is also known as osseous sarcoma that origin from bone. They are much less common In comparison with benign bone tumor and secondary cancer,. It occupies 27th position among all cancer on the basis of occurrence.

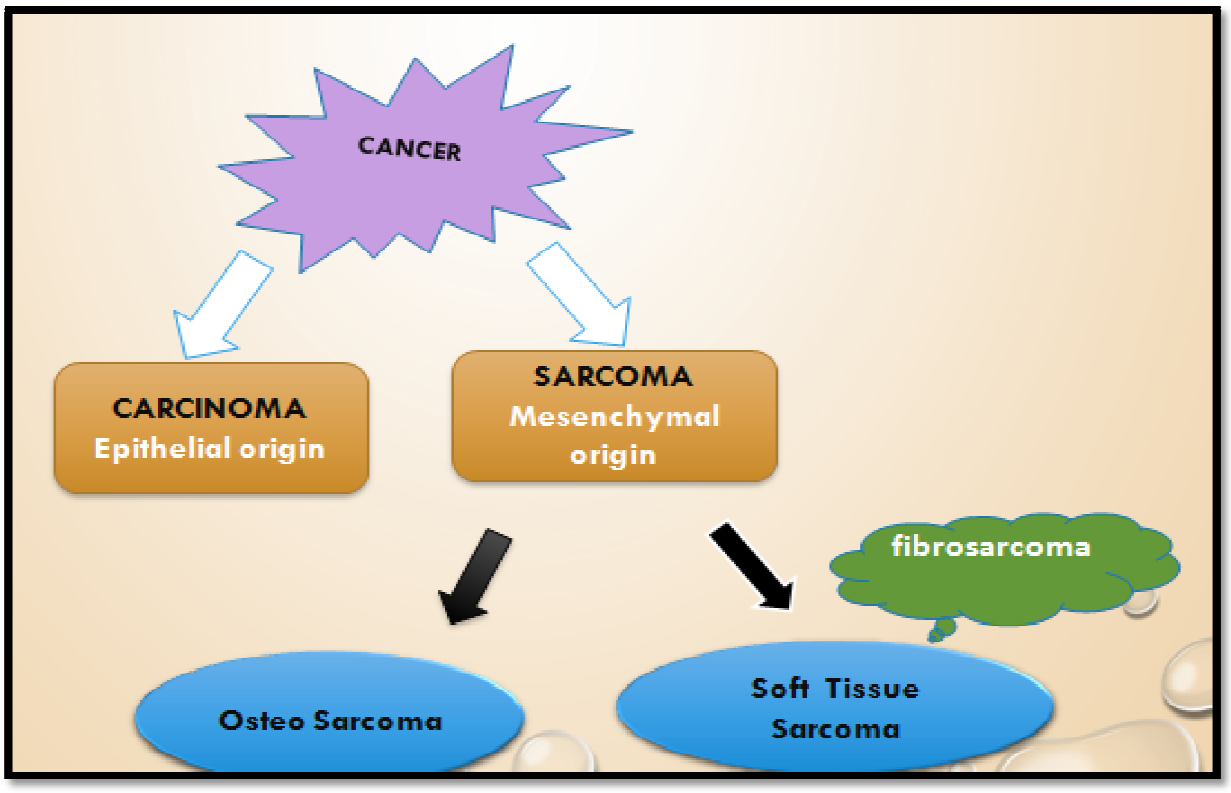


Figure: 1 Types of cancer

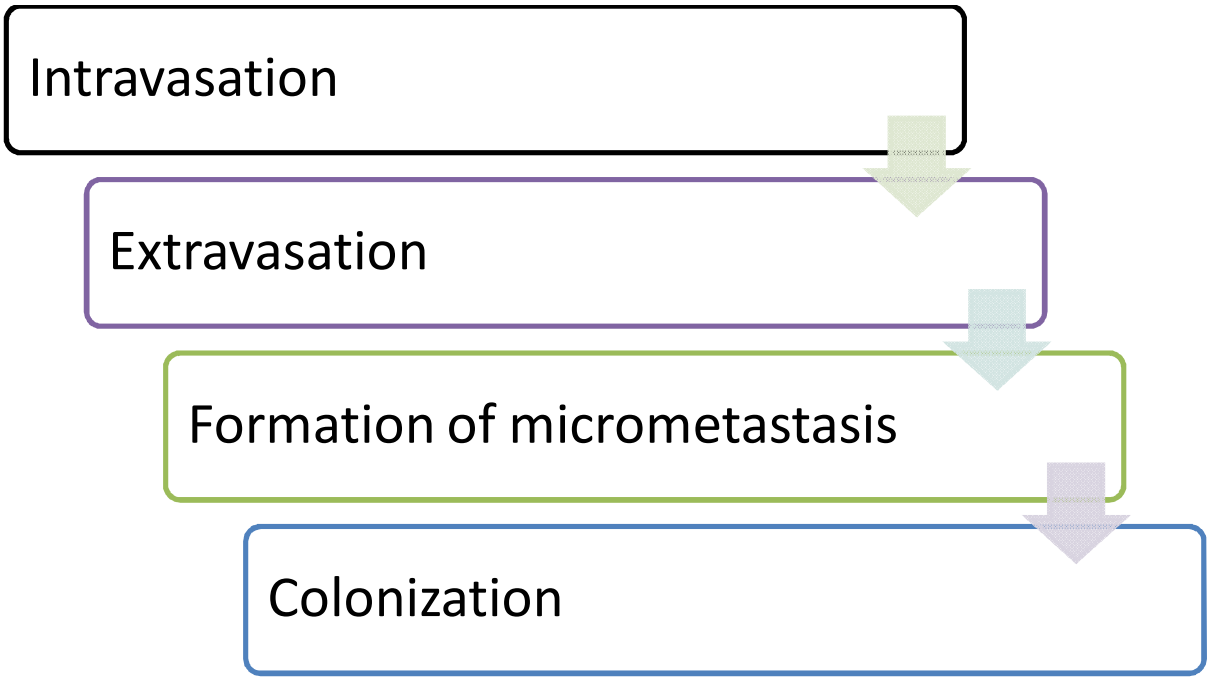
Metastasis is the process by which malignant tumor cells leaves the primary site and migrate to a distance site within the body and establish a secondary tumor . [Chambers AF, Groom AC, MacDonald IC (2002)]. This is the major reason of treatment failure of sarcoma and death of sarcoma patients. It is a very complex process that requires multiple individual steps to establish a tumor at a secondary site. This process involves a change at the molecular level that modifies and disrupts the cell-extracellular matrix interactions and tumor cell–cell interaction. These

molecular changes due to cadherins and integrins signaling through cell adhesion molecules (CAMs) , tissue remodeling through the action of proteinases enzyme like metalloproteinases (MMPs), plasmin, growth factors, and chemokines. These molecules mediate cell processes such as proliferation, migration, invasion, survival and apoptosis [Nguye DX, Bos PD, Massague J (2009)].These mechanisms describe the metastatic cascade in sarcoma that is largely unknown, and which may differ significantly from that of carcinoma, in which there is a preliminary knowledge.

The spread of cancer cells to other parts of the body is depends on:

- Original location of the cancer
- The type of cancer
- The stage of cancer

Metastatic Process



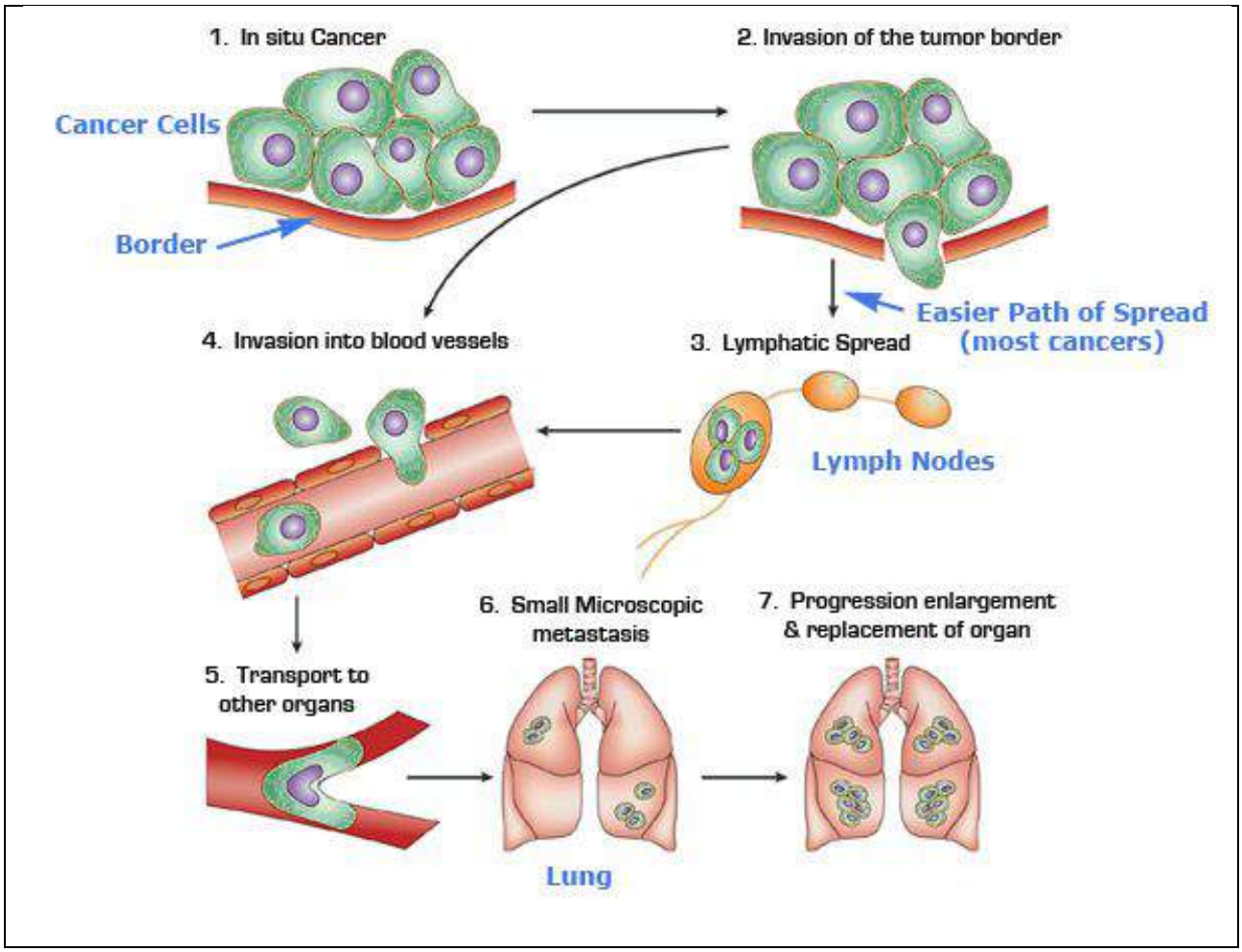


Figure: 2 Different stages of metastasis- 1) Local invasion 2) Intravasation 3) Transport through circulation 4) Extravagation 5-6) Formation of micro-metastasis 6) Colonization.

Metastatic sites

The site where the primary tumor will migrate to and estabilish a colony at distance site or secondary malignant tumor is called the site of metastasis. Metastatic site is organ specific. For organ selectivity, 2 theories are there- Mechanistic theory: According to it the pattern of blood flow determines organ site. Seed and soil theory (Fokas et al. 2007): According to it spreading will occur to that site where there is fertile environment so that compatible tumor cells can grow (Nguyen et al. 2009).

Common site of metastasis are:

Cancer type	**Main site of metastasis**
Bladder	liver, Bone, lung
Breast	brain, liver, lung
Colorectal	lung, peritoneum, Liver,
Kidney	Adrenal gland, brain, liver, lung
Lung	bone, Adrenal gland brain, liver,
Melanoma	brain,liver, lung, Bone, skin/muscle
Ovary	lung, peritoneum, Liver,
Pancreas	Liver, lung, peritoneum

The spread of tumor cells can occur either through lymphatic or blood vasculature leading to metastases to distant organ. Though metastasis is well understood in cancers of epithelial origin (carcinoma), but it is not clearly understood in cancers of mesenchymal origin (sarcoma).

Micro RNAs (miRNA)

MicroRNAs (miRNAs) are the small non-coding RNA, length is about 18–22 nucleotides . They regulate the gene expression in a variety of manners; this includes transcriptional degradation, translational repression, and deadenylation. They binding to 3′untranslated regions (UTR), and coding sequences or 5′UTR of target messenger RNAs (mRNAs) and regulate gene expression posttranscriptional. They are partially complementary to messenger RNA (mRNA) molecules, [V. Ambros, Nature,2004].miRNAs regulate about 30% of protein-coding genome in human.[W. Filipowicz, S.N. Bhattacharyya, N. Sonenberg, Nat. Rev. Genet. 9 (2) (2008).]

The first miRNA was discover in *Caenorhabditis elegans* , and the miRNA is lin-4 ,in 1993 by [Lee R. C. et al.] Till now thousand of miRNAs have been identified in various

organisms by the help of computational prediction, sequencing and random cloning. miRBase, is a data base from where we can retrieve sequence data, miRNA nomenclature, annotation and target prediction information, and hosted by the Sanger Institute.

miRNA biosynthesis

miRNAs encode by genes which are much longer than the processed mature miRNA molecule. Most of miRNAs are present in introns of their pre-mRNA host genes and they share a common regulatory elements, RNA polymerase II are involved in the transcription of miRNAs [Lee Y. et al. EMBO J. 2004,]. By the help of microprocessor complex, The pri-miRNAs are processed in the nucleus, which contain Drosha (RNase III enzyme) [Han, J. et al. Genes Dev. 2004,] and Pasha/DGCR8 a double-stranded-RNA-binding protein, The pre-miRNAs are about 70-nucleotides, and are folded into imperfect stem-loop structures. Then the pre-miRNAs are exported into the cytoplasm with the help of karyopherin exportin 5 (Exp5) and Ran-GTP complex [Yi, R. et al.Genes Dev. 2003]. Ran is essential for the RNA and proteins translocation through the nuclear pore complex [Moore, M. S. et al Nature 1993]. The Ran GTPase forms a nuclear heterotrimer with pre-miRNAswhen it binds to Exp5, After generating of the pre-miRNAs in cytoplasm it finaly form a mature miRNA by undergo an additional processing step with the help of RNAse III enzyme Dicer , a mature RNA is aproximately 22 nucleotides in length. Dicer initiatesto formation the RNA-induced silencing complex (RISC). RISC is responsible for the gene silencing due to RNA interference and miRNA expression [Hammond, S. M. Dicing and slicing: 2003].

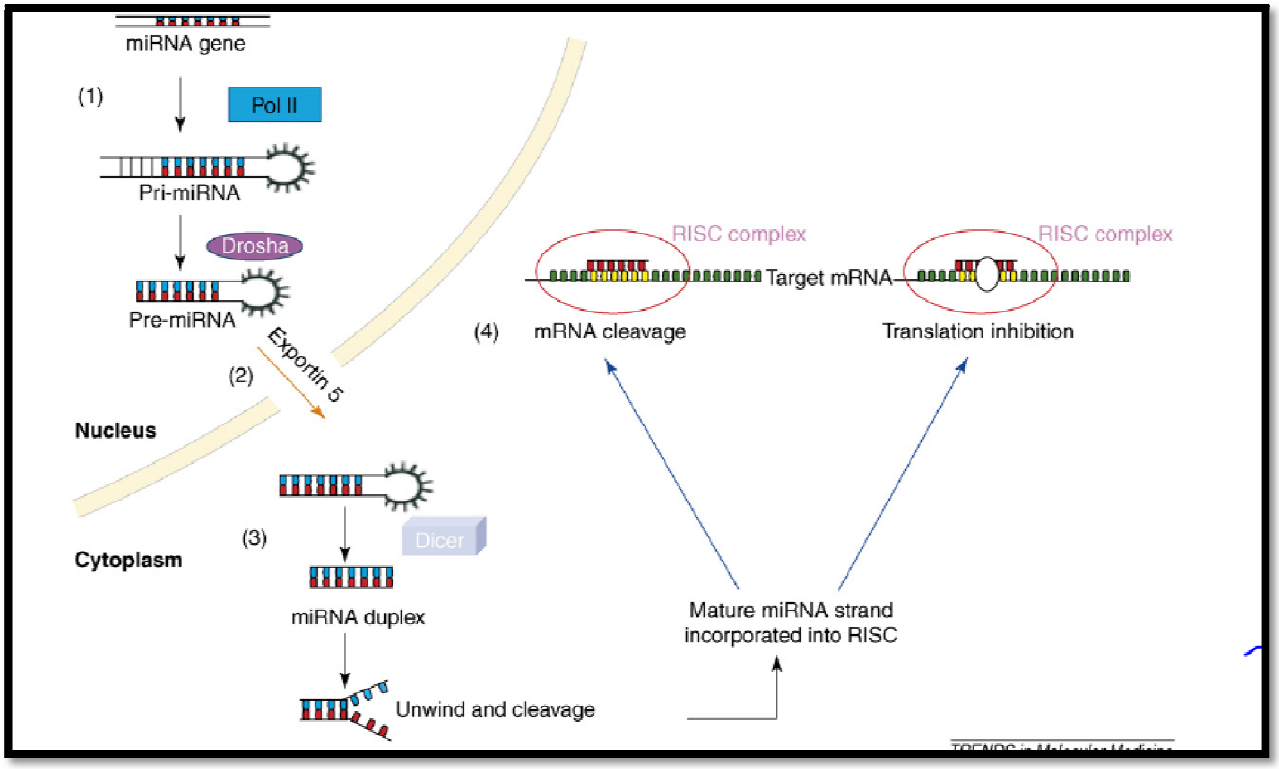


Figure:3 (1) miRNAs are transcribed by RNA polymerase II (pol II) into long pri-miRNA transcripts of variable size, which are recognized and cleaved in the nucleus by the RNase III enzyme Drosha, resulting in an hairpin precursor form called pre-miRNA. (2) This pre-miRNA is exported from the nucleus to the cytoplasm by exportin 5 and is further processed by another RNase enzyme called Dicer (3), which produces a transient 19–24 nucleotide duplex. Only one strand of the miRNA duplex (mature miRNA) is incorporated into a large protein complex called RISC (RNA induced silencing complex). (4) The mature miRNA leads RISC to cleave them RNA or induce translational repression depending on the degree of complementary sites between the miRNA and its target.

miRNA targeting:

miRNA regulate the gene expression via translational repression, transcriptional degradation and deadenylation. They binding to 3′untranslated regions (UTR), and coding sequences or 5′UTR of target messenger RNAs (mRNAs) and regulate gene expression posttranscriptional. They are partially complementary to mRNA molecules, [V. Ambros, Nature,2004]. According to the

Watson and Crick pairing rule towards 5' region of miRNA transcriptional repression miRNA helps in predicting miRNA target which indicate the specific targeting. One-third of the human gene follows the rule to maintain their pairing,

KIAA0101 is a protein coding gene and also known as p^{15PFA} PCNA-associated factor .which is located in chromosome no 15 in human. It is 15-kDa weight protein,and also know as proliferation cell nuclear antigen (PCNA)-associated protein and p15PAF, L5, and OEATC-1. Their main function is to regulate the cell proliferation, cell cycle progression, DNA repair,migration. [Yu P,Huang B, Shen M, et al. 2001]

Over expressed p15PAF, protein helps to protects cells from UV –light (which induced cell death) [Warbrick E. A 2006].Developmental stages of p15PAF protein expression is highly restricted in mouse embryos, [Petroziello J, Yamane A, Westendorf L, et al.2003] But it shows aberrant expression in various cancers, such as brain, breast, kidney, uterine cervix, esophageal hepatic, lung, and colon cancers.[Mizutani K, Onda M, Asaka S, et al.2005]. Some of the clinical studies on p15PAF have supported that it function as an oncogene and induce tumor progression [Guo M, Li J, Wan D, et al 2006]. There are also reports of it functioning as a tumor suppressor in hepatocellular carcinoma [Shanghai Medical College, Fudan University, Shanghai, China. guomingleicn]

Objectives

Objective: 1

Microarray analysis for obtaining differentially expressed genes and miRNAs in Fibrosarcoma.

Objective: 2

Generation of miRNA-mRNA interaction map and prediction of putative novel target pair involved in metastasis of fibrosarcoma

Objective: 3

Experimental validation of selected gene and miRNA expression through qRT-PCR

Objective: 4

To study of role of miRNA in metastasis of fibrosarcoma via different in vitro assays

MATERIAL METHODS

TOTAL FLOWCHART OF EXPERIMENT

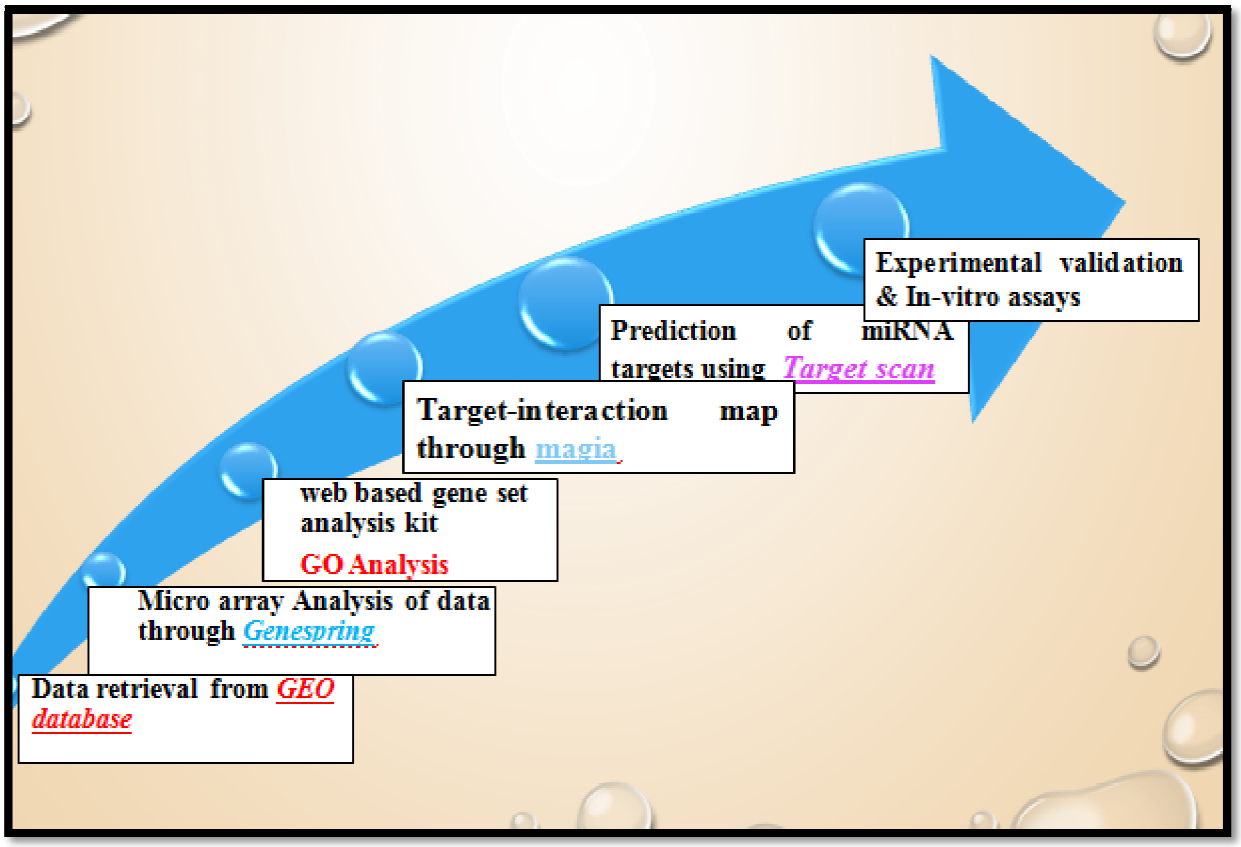


Figure: 4 flow chart of experiment

Gene expression data

For gene expression analysis of mRNA and miRNA in order to study the differentially expressed genes/miRNA in control and diseased sample in fibrosarcoma expression data is taken from Gene Expression Omnibus (GEO) data base.

From GEO data base we can retrieve sequencing, microarray, and next generation sequencing data.It supports MIAME submissions that collects and provides freely next generation sequencing data, microarray data and high throughput functional genomic data which is submitted by the scientific community.

For our analysis we had collect the sample, miRNA and mRNA expression data of the fat sample as a control and fibrosarcoma as a test sample.

PLATFORMS taken-

Expression Data	GEO accession	Title
mRNA	GPL96	Affymetrix Human Genome U133A Array
miRNA	GPL8179	Illumina Human v2 MicroRNA expression beadchip

Samples taken:

Expression Data	GEO accession	Title
mRNA	GSE21122	Whole-transcript expression data for soft-tissue sarcoma tumors and control normal fat specimens
miRNA	GSE36982	MicroRNA profiling of primary high-grade soft tissue sarcomas and control normal fat

2) Microarray analysis of gene Expression Data:

A) Gene expression analysis:

- Gene expression analysis is done By using Agilent's GeneSpring GX 12.6 software.
- All samples collected from GEO data base were consider for further analysis
- (fibrosarcoma and normal control fat present in the GSE was taken for analysis.)

Table 3. Total Number of samples taken for mRNA and miRNA expression analysis

Gene expression data	Name of sample	No of samples
mRNA	Normal fat	9
mRNA	MFH:Myxofibrosarcoma	31
miRNA	Myxofibrosarcoma	8
miRNA	Normal fat	2

B) miRNA expression analysis:

miRNA expression analysis was done using GeneSpring, using a miRNA specific platform such as Illumina. For further analysis we consider only those miRNAs which are differentially expressed greater than 2 fold between the control and test samples .

C) Analysis of Gene and miRNA list:

To finding a probable oncogene the gene ontology analysis was carried out, for the genes which are up-regulated using web based gene set analysis GO analysis (gene ontology). Genes with functions and probable role in metastasis was further focused on.

1) Target interaction map through Magia2 Software:

This study was carried out to display miRNA –Mrna interaction. In a given biological context. A set of gene is designed to detect consistent effects of microRNAs on the joint expression of multiple targets. In a single test, the association between microRNA expression and target gene set expression, and the contribution of the individual target genes in the association are determined.

IN VITRO VALIDATION:

A) Cell Line and Culture

For in vitro cell culture Fibrosarcoma cell line HT-1080 was used which was collected from National Centre for Cell Science, Pune, as a test sample.Normaly it is culture in DMEM (Dulbecco's Modified Eagle'sMedium) growth media , which contain high L-glutamine supplemented with 10% fetal bovine serum, glucose and 1% penstrep antibiotic solution were used. It was incubated in CO2 incubator, maintained at 5% CO2 concentration and 100% humidity. According to logarithmic growth all experimental analysis was done with cells. The media contains a ph indicator, phenol red, and the color of medium changes when the media is utilize by cells,it change from red to orange ,finally to pale yellow because of change in pH.

- WI-38 was collect from Bose Institute Kolkata, as control sample.

B) <u>RNA ISOLATION:</u>

RNA extraction from HT-1080 and WI-38

- **mRNA isolation:**
  For mRNA isolation HIMEDA kit (HiPurA^{TM}Total RNA minipre Purification Kit) was used according to the manufacturer's instructions.

Procedure

1) We had harvested a maximum of 1×10^7 cells and appropriate volume of RLT buffer was added.

2) 1 volume of 70% ethanol was added to the lysate, and mixed well by pipetting.

3) 700µl of sample including any precipitation was transferred to an RNeasy mini spin column Placed in 2ml supplied collection tube. Then the lid was closed and centrifuged for 15 sec at ≥8000xg.Then discarded the flow through.

4) To RNeasy spin column 700µl of RW1 was added. Then the lid was closed and centrifuged for 15 sec at 8000xg.Then flow through was discarded.

5) To RNeasy spin column 500µl of RW1 was added. Then the lid was closed and centrifug for 15 sec at ≥ 8000xg.Then flow through was discarded.

6) To RNeasy spin column 500µl of RW1 was added. Then the lid was closed and centrifuge for 2 minute at ≥ 8000xg.

7) In a new 1.5ml collection tube the RNeasy spin column was placed. 30-40µl RNase free water was added directly to spin column membrane. Then the lid was closed and centrifuged for 1 minute at ≥ 8000xg in order to elute RNA.

8) If the expected RNA yield is more than 30µg, then the step-7 was repeated using another 30-50µl of RNase free water or using the elute from step-7.The collection tube were reused from Step-7.

9) By using Eppendorf Nan Drop, the purity and yield of RNA was measured. It is a curette free spectrophotometer eliminating the need for other sample containment device and also allows for clean up in seconds. 1µl of the sample can be measured with this instrument with high accuracy and reproducibility. 1µl of sample was pipette onto the end of a fiber optic cable. A second fibrotic cable is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. A pulsed xenon flash lamp provides the light source and spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample.

C) Micro RNA (miRNA) isolation:

For miRNA isolation from HT1080 and WI-38 mirVana^{TM} Isolation kit from Ambion was used.

1) Cells were collected after trypsinization. And then wash with PBS and cells pellets were collected, then it was kept in ice for some minute.

2) PBS was removed, 600 µl lysis or binding solution was added. (If the cells are 100s in number -300 µl)If cells are 1000s -600 µl)

3) Now the solution was vortex vigorously to make homogenous lysate.

4) 1/10th (for 600/10=60 µl) of microRNA homogenate additions was added to cell, then mixed Well by vertaxing or inverting several times , then kept on ice for 10 minute.

5) 1 volume of Acid phenol chloroform that is equal to lysate volume is added before addition of miRNA homogenate, then vortexed for 30 to 60 sec in order to mix then it was centrifuged for 5 min at 10,000 g at room temperature to separate aqueous and organic phases. (After Centrifugation the interphase should be compact if it is not then centrifugation is repeated)

6) Aqueous phase is carefully removed without disturbing the lower phase and transferred into a new tube.

7) 1.25 volume of room temperature 100% ethanol was added to aqueous phase.

8) A filter cartridge was placed into one of the collection tubes, then it was mixed by pipetting then centrifuged for 15 sec at 10000 rpm, then flow through was discarded until all the lysate is through.

9) 700µl of miRNA wash solution I was added to filter cartridge and then centrifuged for 5 to 10 sec and then flow through was discarded.

10) 500 µl of wash solution 2/3 centrifuged it for 5 to 10 sec. Again repeat it with 500 µl of wash solution 2/3.

11) After discarding the flow through from last wash, the filter cartridge was replaced same in collection tube and assembly was undergo spinning for 1 min to remove residual fluid from

12) Filter cartridge was transferred into a fresh collection tube .100µL of pre heated (95oC) elution buffer or nuclease free water was applied ,cap was closed and it undergo spinning for 20 to 30 sec at maximum speed to remove microRNA.

13)The elute was collected and stored at -20^{o}C or below.

D) <u>cDNA SYNTHESIS</u>

- **cDNA Synthesis of mRNA**

Total RNA was reversely transcribed using an Invitrogen's first-strand cDNA Synthesis Kit using oligo dT primer in a total volume of 20 µl. Before use as a template for PCR amplification the cDNAwas stored at −80°C.

1) Each of the components were mixed and centrifuged briefly before use.
2) For each reaction the following in a sterile 0.2 or 0.5 tube was combined.

Component	**Amount**
RNA (2ug)	4 µl
10mM dNTP mix	1 µl
Primer - oligo (dT)	1 µl
DEPC treated water	4 µl

3) The RNA/Primer mixture was incubated at 65°C for 5 minute and then placed on ice for atleast 1 minute.

Component	1RXn	10RXn
10X RT buffer	2 µl	20 µl
25Mm Mgcl2	4 µl	40 µl
0.1MDTT	2 µl	20 µl
RNase out (400/µL)	µl	10 µl

4) Following 2X reaction was prepared in a separation tube by adding each component in the indicated order.

5) To each RNA/Primer from step 3, 9µl of 2X reaction mixture was added, mixed gently collected by a brief centrifuge.

6) It was incubated at 42°C for 2 minute.

7)1µl of superscript TM II RT was added to each tube.

8) It was incubated for 50 minute at 42oc.

9) The reaction was terminated at 70oc for 15 minute and then chilled on ice.

10) By brief centrifugation the reaction was collected. To each tube 1 µl of RNase H was added and incubated at 37oc for 20 minute and then the reaction was used for PCR immediately.

- **cDNA Synthesis of miRNA :**For cDNA synthesis an invitrogen kit (NCode^{TM}VILO^{TM} miRNA cDNA Synthesis kit was used.

  1) 100 to 1µg of optimal RNA was taken.

  2) DNase I may be used to eliminate genomic cDNA contain.

  3) Undiluted cDNA are to be used for qRT-PCR.

  4) The following reaction volume may be scaled as needed upto 100µl .For single reaction, combine the following components in a tube on ice. For a multiple reactions a master mix is prepared without RNA.

Component	Amount
5X reaction mix	4 µl
10X superscript	2 µl
Total RNA (1ug)	2.5 µl
DEPC treated water	11.5 µl

  5) The tube was capped then gently vortexed to mix and centrifuge then briefly.

  6) The tube was then incubated at 37°C for 60 minute, terminated at 95°C for 5 minute and then hold the reaction at 4°C until use.

QUANTATIVE RT-PCR ANALYSIS:

It is used to amplify and simultaneously detect and quantify the targeted RNA molecule.

Two common methods are there for qRT-PCR:

- Non-specific fluorescent dyes that is intercalate with any double-stranded DNA, such as SYBR green.
- Sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary sequence to (mRNA) and non-coding RNA in cells or tissues.

We used SYBR green dye as a florescent dye.

1) Procedure of mRNA qRT-PCR

To perform PCR by using RNA as a template, it must first have to transcribed into cDNA by a reverse transcription reaction, in which cDNA is act as template for RT-PCR with gene specific primers.

Table No 4. Primer name and sequence with length and its amplicon size

Primer name	**Sequence**	**Amplicon size**
ACTB	F- F-CATGTACGTTGCTATCCAGGC	**250**
	R- R-CTCCTTAATGTCACGCACGAT	
p15PAF	F- F-ATGGTGCGGACTAAAGCAGAC	**123**
	R- R-CCTCGATGAAACTGATGTCGAAT	
miR-429	F-CGGCGTAATACTGTCTGGTAAAACCGT	**22**

In Eppendorf Master plex Real Time PCR, RT-PCR was carried out. Beta actin (ACTB) was used as endogenous control. Primer concentration was normalized and gene specific forward and reverse primer pair was mixed. Each primer that is forward and reverse primer concentration in mixture was 3.5µl.

a) Now the experiment was ready and the following PCR program was made on. A copy of the set up file was saved and all other PCR cycles were deleted. Threshold frequency was taken was 33%. The temperature cycle were taken as follows:

Stages	Temperature (^{o}c)	Time	Cycle
Stage 1	95	20 sec	1
Stage 2	95 55 68	15 sec 15 sec 20 sec	40
Stage 3	95 60 95	15sec 15sec 15sec	1

b) cDNA was diluted to 1:20 ratio concentration to use 20ng per reaction and then primer was added.

c) 10µl of a real time PCR reaction volume was made.

Reagent	**Amount**
SYBR Green Mix(2x)	35µl
cDNA stock(cDNA:dH2O[1:20])	40 µl
Primer pair mix(3.5µl each primer)	7 µl

d) In each optional tube as the following mixture was made follows.

e) With the help of in-built software , the RT-PCR result was anal

f) The tubes were removed from the machine, after PCR is finished.

2) <u>Procedure of miRNA qRT-PCR</u>

a) Undiluted cDNA was used for quantification.

b) 10µl of a real time PCR reaction volume was made.

In each optional tube as the following mixture was made follow

Reagent	**Amount**
SYBR Green Mix(2x)	5 µl
cDNA stock	2µl
DEPC H2O	2.6 µl
Forward primer	0.2 µl
Reverse Primer	0.2 µl

c) With the help of in-built software, the RT-PCR result was analyzed.

d) The tubes were removed from the machine, after PCR is finished.

C) miRNA Over expression:

miRNA over expression was done by transfection of miRNA mimic, obtained from Sigma. It contains artificial miRNA-like RNA fragments and mimics a natural double stranded miRNA molecule.

miRNA mimic Transfaction.

miRNA mimic and scramble at 40 nM concentration was transfected into HT-1080 Cell line using Lipofectamine 2000 Invitrogen, according to the manufacturer's instructions.

D) qRT-PCR of mimic treated gene

Same mRNA and miRNA isolation procedure is followed for mimic treated cell and non treated gene.

E) Proliferation assay

Cells were transfected with a miR-429 mimic using Lipofectamine 2000 (cat# 11668-027, Invitrogen) according to the manufacturer's instructions. The cell proliferation assay was done with the 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays. Cells were seeded into 96-wells plates for MTT analysis. Twenty-four hours after cell transfections, 20 μL of MTT (5mg/mL) was added into each well and incubated for 4 hours. After that, the supernatant was discarded, and then 150 μL dimethyl sulfoxide was added to each well and oscillated for 10min to dissolve the precipitate. Finally, OD absorbance (at 570 nm) was measured using UV spectrophotometer at 24 hours, 48 hours, and 72 hours after transfection. The assay was performed three times in triplicates.

D) Scratch assay or Wound healing assay

The scratch assay allows studying cell migration and cell interactions.

1. Seed the cells in multi-well plates and culture until confluent. Using a pipette tip make a straight scratch, simulating a wound.
2. We had make a scratch keeping the pipette tip under an angle of around 30 degrees to keep the scratch width limited, and which is suitable for imaging of both wound edges .

Scratch assay is suitable for studies on the effects of cell-cell interactions, cell-matrix interaction, on cell migration, mimic cell migration during wound healing.

Result

1. **mRNA Expression analysis** : There are 1068 differentially expressed gene were obtain from miRNA expression analysis between Myxofibrosarcoma and the normal fat samples, out of these 558 gene are up regulated and 510 gene are down regulated. This Hierarchical clustering showed that relatedness among test and control sample.

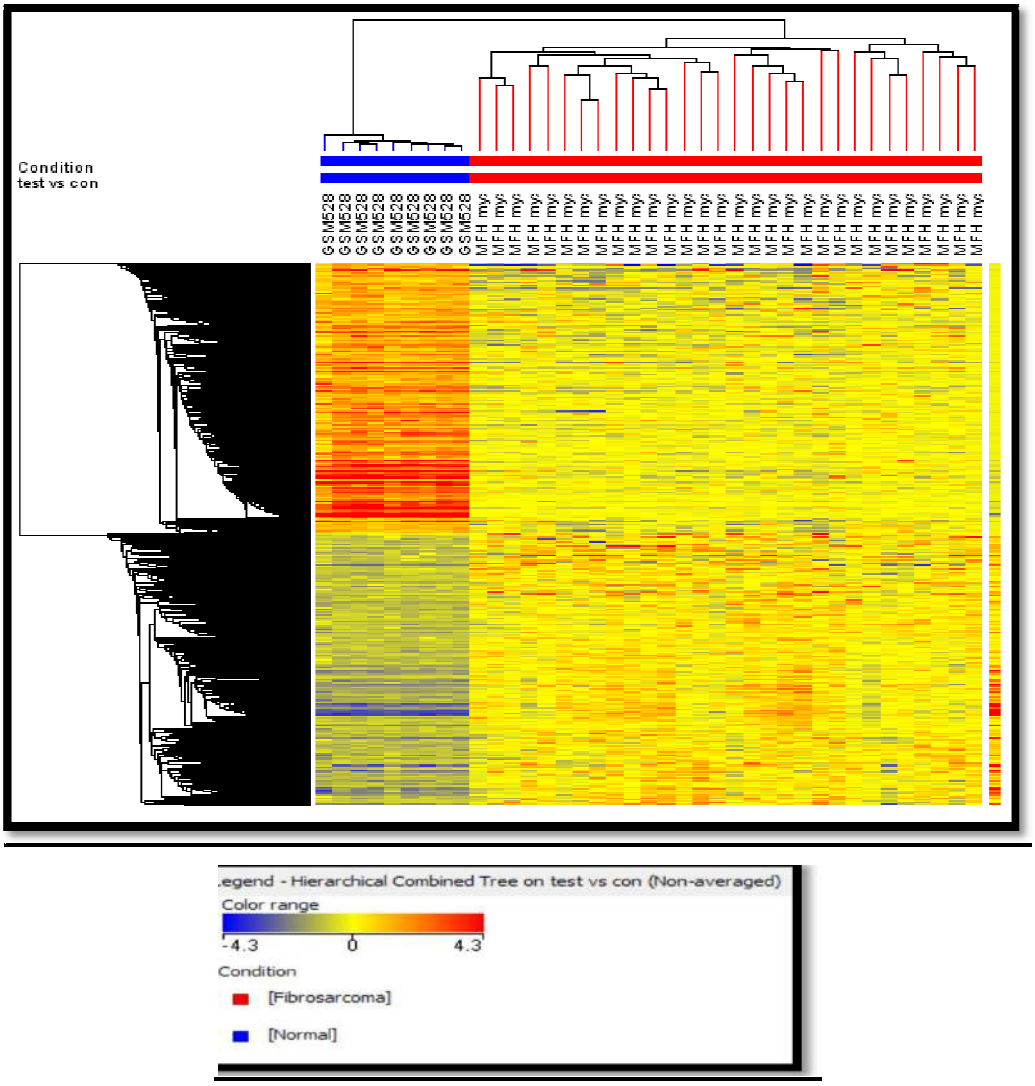


Fig 5: Hierarchical clustering of MFS and normal fat samples

2. **microRNA Expression Analysis:**

There are 77 differentially expressed miRNAs were obtained between Myxofibrosarcoma and the fat samples. From these 51 miRNA were up-regulated and 27 miRNA wear down-regulated.

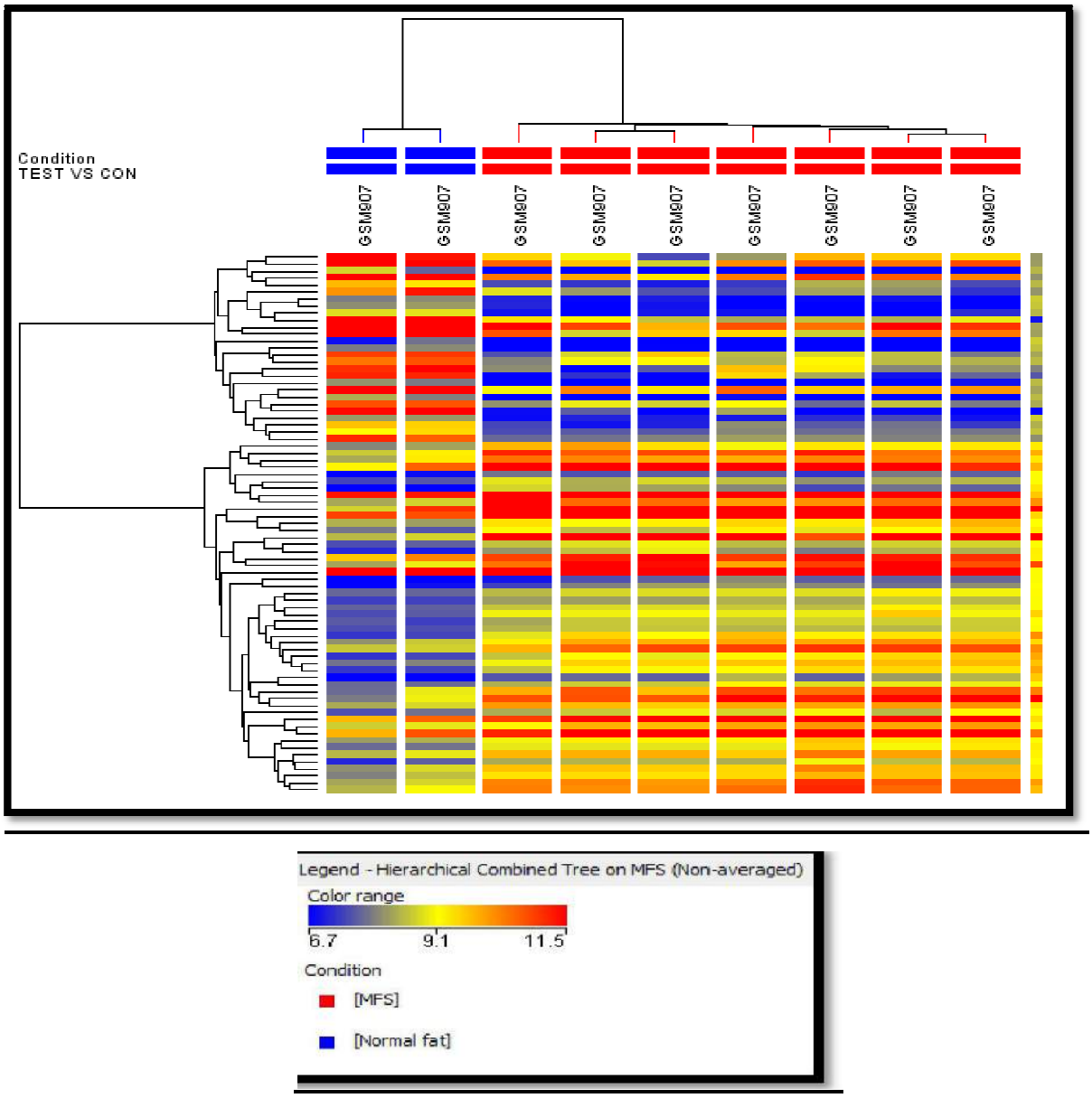


Fig 6: Hierarchical clustering of differentially expressed miRNA.

3. Target interaction map analysis:

The Target interaction map resulted shows that 36 genes targeted by the miR-429 and 4 gene target by miR-37.

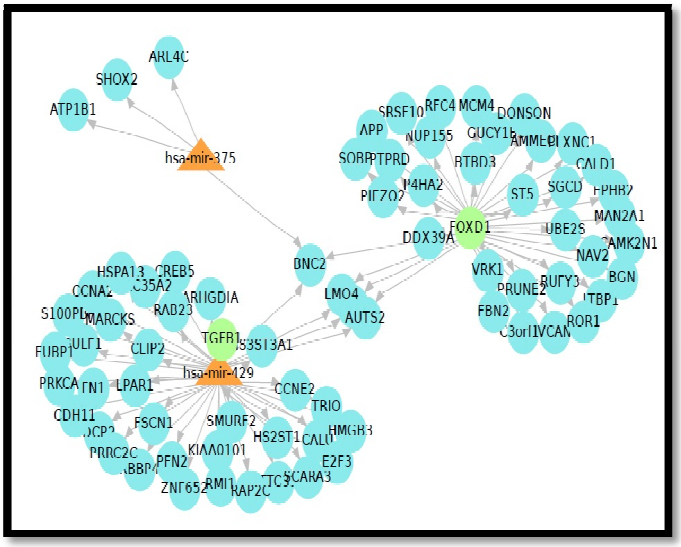


Figure: 7 Interaction map Analysis of mRNA and miRNAs involved in fibrosarcoma

4. RNA hybrids:

This result shows that KIAA0101 form hybrid with hsa-miR-429 at position 22067 and Eight nucleotide pairing (8 mer) and form a thermodynamically stable binding having (Minimum free energy) Mfe -15.2kca/mol. Due to which we select miR-429-and KIAA0101 as novel pair and responsible for metastasis in fibrosarcoma.

```
TARGET : gi|568815583:c64387687-64364994
length: 22694
MIRNA  : hsa-miR-429
length: 22

mfe: -15.2 kcal/mol


position  22067
target 5'     U       A  UUAAAAAAUU          A 3'
                UUUUGC CA            CAGUAUUA
                AAAAUG GU            GUCAUAAU
miRNA  3' UGCC          CU                    5'
```

5) Quantative RT-PCR analysis: of 6 KIAA0101 Expression in HT1080 vs WI-38

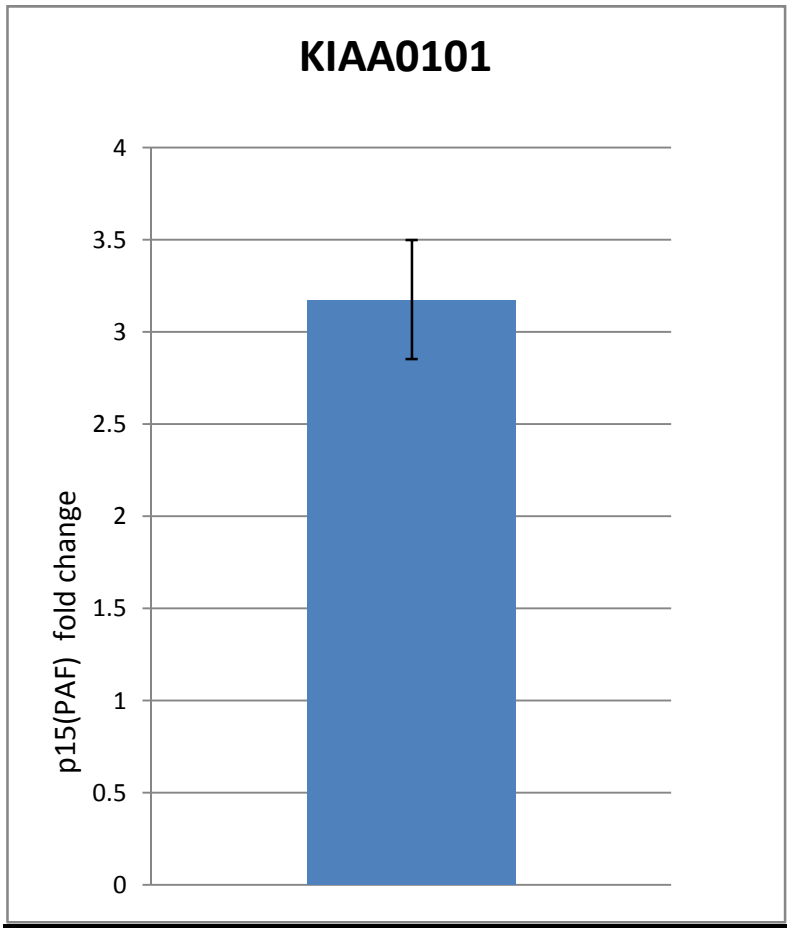


Figure:9 P15 (PAF) Expression in HT1080 vs WI-38

The real time PCR shows that Expression of p15(PAF) is 3.5 fold up regulate in HT-1080 as compaire to control sample WI-38.

6) Quantitative PCR data analysis of miRNA in HT1080 vs WI-38

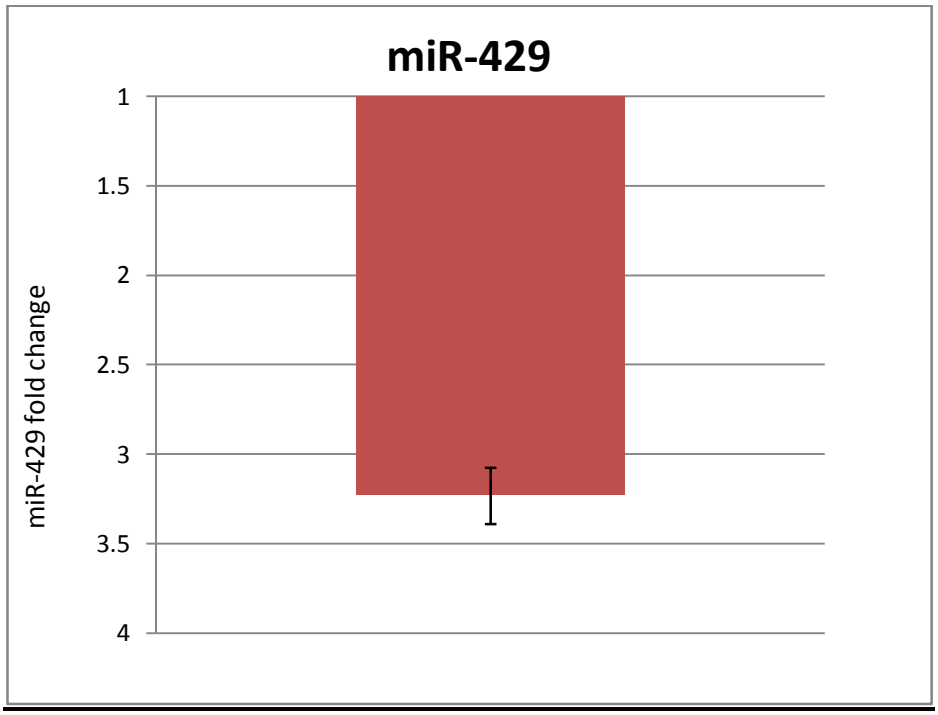


Figure :10 miRNA in HT1080 vs WI-38

The real time PCR shows that Expression of miR-429 is 3.25 down regulate in HT-1080 as compaire to control sample WI-38.

7) **Quantative RT-PCR data analysis of miRNA expression after mimic treatment**

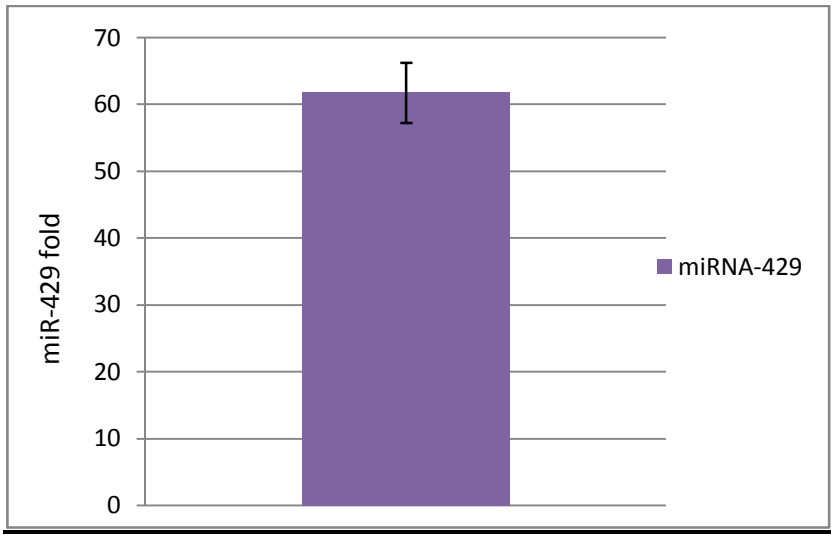


Figure 11: miRNA expression after mimic treatment

Post mimic treatment the expression of miR-429 is 62 fold up regulated. This showed that the miRNA transfected was stably over expressed.

8) **qRT-PCR data analysis : KIAA0101 Expression post miRNA treatment**

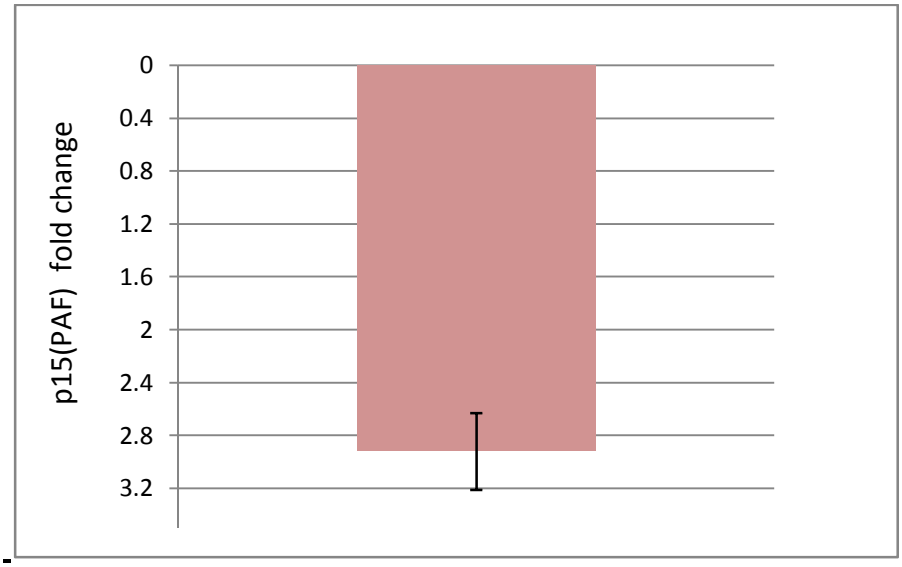


Figure: 12 P15 (PAF) Expression post miRNA treatment

After mimic treatment expression of mRNA is 3.2 fold down regulated. Over expression of miRNA suppress the expression of mRNA .

9) Proliferation assay

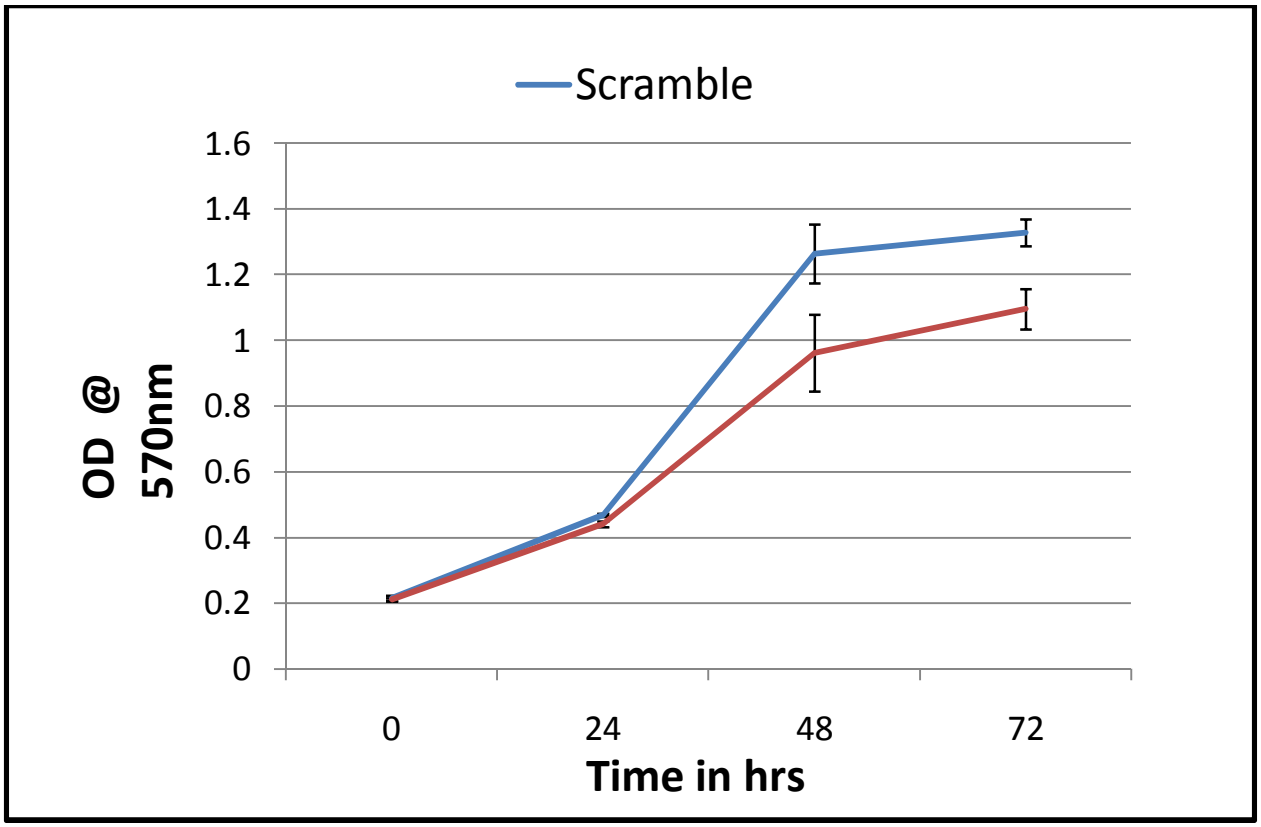


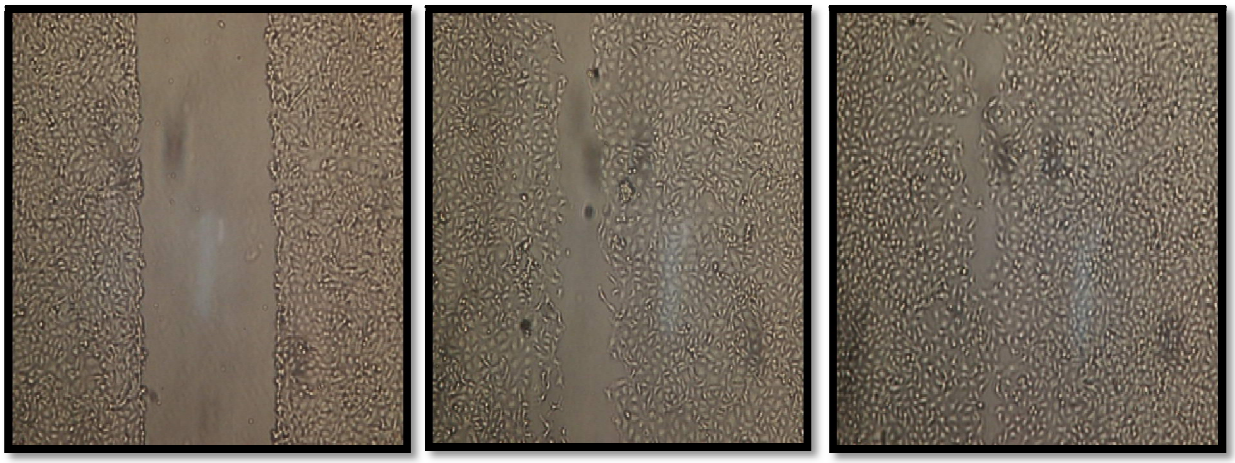
Figure: 13 cell viability at different time durition

After transfect ion of miRNA mimic, cell viability is less in mimic treated cell. It indicate that mimic inhibits to proliferation of HT-1080 cell, whereas scramble doesn't inhibit. At 0 hrs cell viability is same on both mimic treated and scramble treated is 0.2 at OD 570nm, but at 24hrs cell viability is 0.41 in mimic treated and 0.43 in scramble, At 48hrs and 72 hrs cells viability or proliferation rate is more in scramble as compare to mimic treated miRNA.

Scratch assay

Scramble Treated cell

0 hrs **12 hrs** **24 hrs**



1) **miR-429 mimic treated**

0 hrs **12 hrs** **24 hrs**

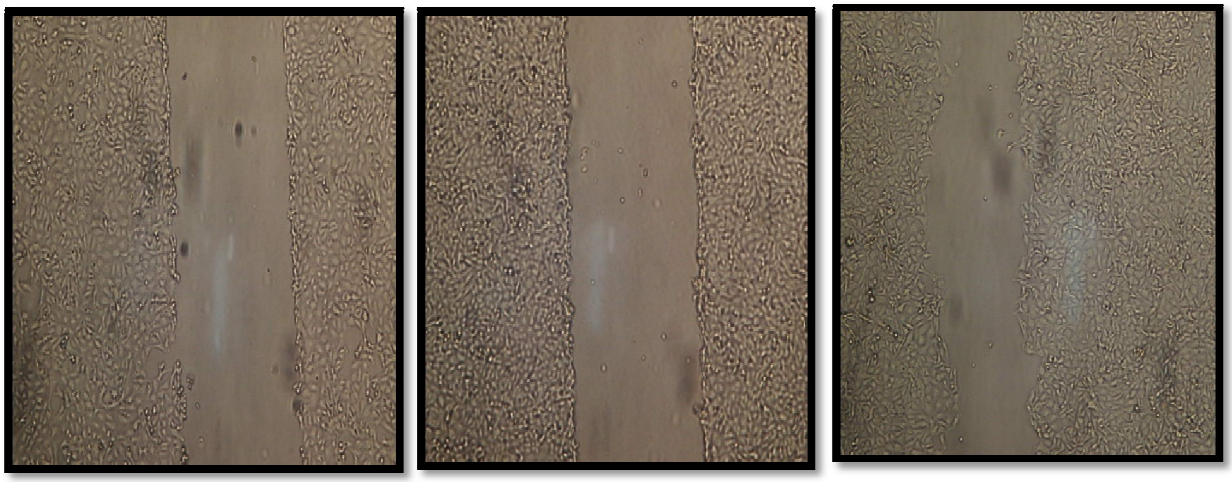


Figure 14: scratch assay of mimic treated cell and scramble treated cell

Scramble treated HT-1080 cells migrate first to fill up the scratch as compare to mimic treated HT-1080 cells. Mimic treated cells are inhibited by miRNA mimic due to which HT-1080 cell restrict to fill up the scratch. And conclude that miRNA mimic inhibit the migration, by acting a tumor suppressor.

Conclusion:

miR-429 expression is decreased in Fibrosarcoma where it might be acting as tumor suppressor miRNA in cellular model, Where it prevents tumor development by negatively inhibiting oncogene KIAA0101 that induce cell proliferation, cell migration. Over expression of miR-429 inhibits the cell proliferation and cell migration which might be helping in metastasis and ultimately contributing to lethality of Fibrosarcoma. From the emerging evidence of our project miRNAs function tumor suppressors, and regulate mRNA expression. Transfection of miRNAs to regulate metastasis of fibrosarcoma , artificial miRNAs could be synthesized to down-regulate the function of miR-429 and prevent the formation of metastasis by inhibiting cell proliferation and cell migration. Thus, artificial miRNAs can be designed to block the expression of these tumor suppressor gene based on the complementary characteristics of miRNAs to their targeted mRNAs. From the evidence suggests that miRNAs play important roles in metastasis of fibrosarcoma. Some miRNAs may be directly involved in fibrosarcomsarcoma development by controlling cell differentiation and cell migration, while others may be involved in sarcoma by targeting sarcomas oncogenes and/or tumor suppressors. Understanding of the Function of miRNAs is providing the new way on the molecular basis of fibrosarcom, and new biomarkers for diagnoses and sarcomas therapy.

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